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Simple Assay of Calcium Dependency for Virulent Plasmid-Bearing Clones of *Yersinia enterocolitica*

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A simplified procedure to detect the calcium dependency of virulent plasmid-bearing strains of *Yersinia enterocolitica* was developed. A low-calcium, agarose-based medium of brain heat infusion with added magnesium effectively differentiated plasmid-bearing and plasmidless isolates. Further, the expression of calcium dependency in plasmid-bearing strains of *Y. enterocolitica* as measured by the average colony diameter was proportional to the calcium concentration of the assay.

Strains of *Yersinia enterocolitica* that are pathogenic in humans harbor a specific plasmid DNA of about 40 to 48 megadaltons which is involved in virulence, calcium dependency (CAD), and expression of temperature-dependent properties (4, 5, 8, 10). At 37°C, in the presence of magnesium, plasmid-bearing strains (P^+) form pinpoint colonies on a calcium-deficient medium, whereas plasmidless strains (P^-) produce large colonies regardless of calcium level (1, 7). CAD is routinely monitored by the use of magnesium oxalate (MOX) agar (9); however, MOX agar is both complex and laborious to prepare. Moreover, since oxalate is used to complex with calcium (8), there is a possibility that other essential minerals may be sequestered (6), thereby affecting bacterial behavior. The purpose of the present study was to develop a simple low-calcium plating medium suitable for CAD testing that did not require use of a chelating agent. The method is based on the observation that agarose contained less calcium than agar.

The *Y. enterocolitica* strains used in this study are listed in Table 1. Detailed descriptions of the strains and sources are given elsewhere (3). The presence of plasmid DNA in bacteria was determined by the method of Bhaduri et al. (2).

TABLE 1. Virulence and virulence-associated properties of plasmid-bearing strains of *Y. enterocolitica* and their plasmidless derivatives

Strain	Serotype	CAD	Crystal violet binding	Autoagglutination	Hydrophobicity	Diarrhea[a] in mice	Plasmid (40–45 MDa[b])
GER	O:3	+	+	+	+	+	+
GER-C	O:3	−	−	−	−	−	−
EWMS	O:3	+	+	+	+	+	+
EWMS-C	O:3	−	−	−	−	−	−
PT18-1	O:5, O:27	+	+	+	+	+	+
PT18-1-C	O:5, O:27	−	−	−	−	−	−
O:TAC	O:TACOMA	+	+	+	+	+	+
O:TAC-C	O:TACOMA	−	−	−	−	−	−
WA	O:8	+	+	+	+	+	+
WA-C	O:8	−	−	−	−	−	−

[a] Fecal material consistency was liquid; diarrhea was observed on days 3, 4, and 5 postinfection.
[b] MDa, Megadaltons.

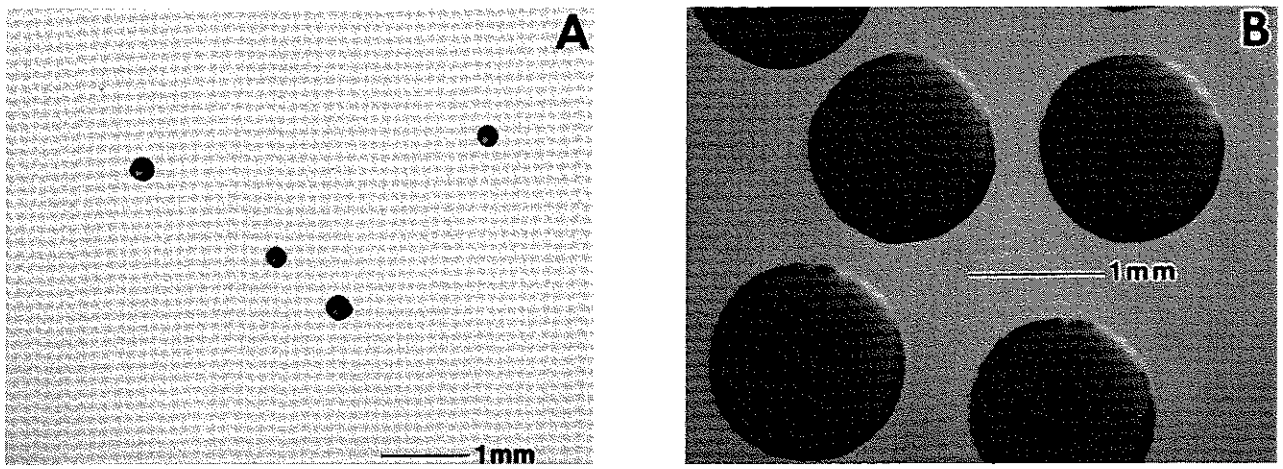

FIG. 1. CAD test response of colonies of *Y. enterocolitica* GER (serotype O:3) when cells were grown on BHO for 24 h at 37°C. (A) Pinpoint colonies (0.36 mm in original diameter) of virulent P^+ cells. (B) Large colonies (1.37 mm in original diameter) of avirulent P^- cells.

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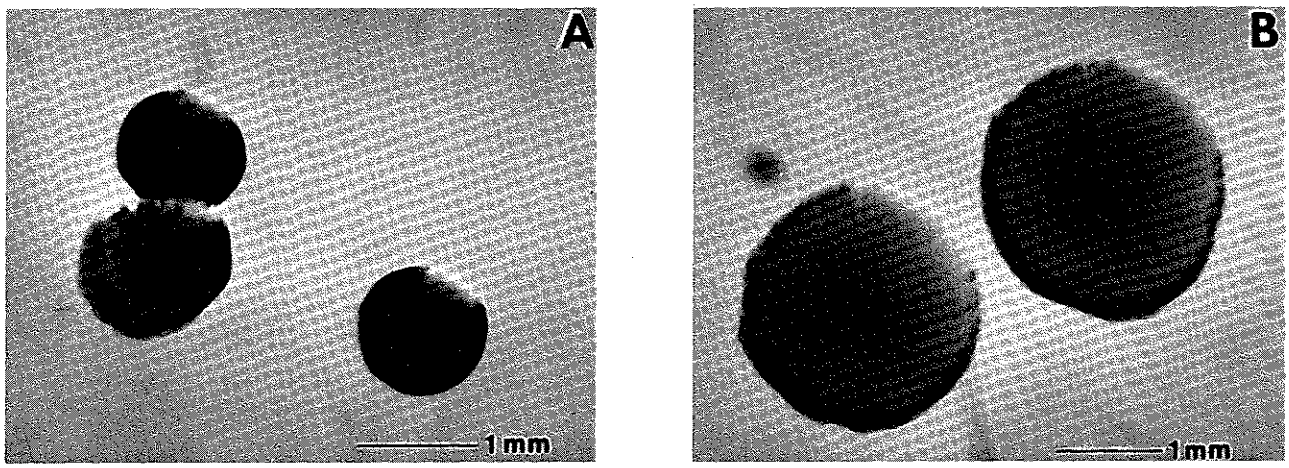

FIG. 2. Colony morphology of *Y. enterocolitica* GER (serotype O:3) when cells were grown on BHA for 24 h at 37°C. (A) Small colonies (1.13 mm in original diameter) of virulent P^+ cells. (B) Large colonies (2.4 mm in original diameter) of avirulent P^- cells.

Crystal violet binding, autoagglutination, hydrophobicity, and mouse virulence tests were performed as previously described (2).

Brain heart infusion (BHI) agarose (BHO) was prepared by adding agarose type V (Sigma Chemical Co., St. Louis, Mo.) to a final concentration of 1.2% to BHI broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.1% magnesium chloride. BHI agar (BHA) (Difco) was prepared as recommended by the supplier. Since BHI is the common ingredient in these media, the amounts of calcium present in agar and agarose determine the concentrations of calcium in BHA and BHO, respectively. The concentrations of calcium in BHI broth, BHA, and BHO were measured by atomic absorption analysis (11); the calcium content was comparatively high in BHA (1,500 μM) but low in BHI broth (245 μM) and BHO (238 μM). The CAD test was performed with calcium-deficient BHO and compared with a CAD test performed with calcium-deficient MOX agar prepared as described by Higuchi and Smith (9). As a control, BHA was used as a calcium-adequate medium. P^+ and P^- cells were grown separately in BHI broth for 18 h at 25°C with shaking.

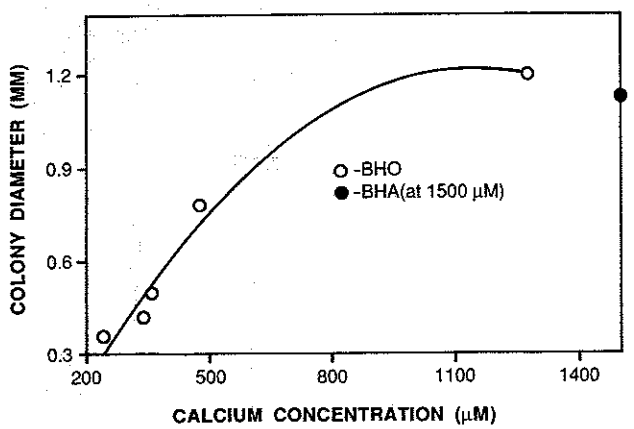

FIG. 3. Effect of exogenously added calcium in calcium-deficient BHO on colony size of P^+ cells of *Y. enterocolitica* GER (serotype O:3). During preparation of BHO, calcium chloride was added exogenously to final concentrations of 338, 358, 476, and 1,276 μM. The data presented here are the results of one of three experiments; however, all showed similar patterns.

Cells were diluted to 10^3/ml (2) and surface plated on BHO, MOX agar, and BHA. After the plates were incubated at 37°C for 24 h, colony morphology was examined. The colony diameter was determined by using a calibrated filar micrometer eyepiece (Bausch & Lomb, Inc., Rochester, N.Y.) on 10 colonies from each medium.

We observed that when the P^+ strains were cultivated at 37°C on BHO and BHA, two colony types were readily discernible after 24 h. On BHO, P^+ cells formed pinpoint colonies 0.36 mm in diameter (Fig. 1A), whereas the P^- cells formed much larger colonies (1.37 mm in diameter) (Fig. 1B). When MOX agar was used as the calcium-deficient medium, colonial morphologies of P^+ and P^- strains were similar to those reported by other investigators (1, 7). The colonies of P^+ cells on MOX agar were smaller than those on BHO after the same period of incubation, suggesting that oxalate may either be toxic or bind other essential minerals (6). On calcium-adequate BHA, colonies of P^+ cells were smaller (diameter, 1.13 mm) (Fig. 2A) than colonies of P^- cells (diameter, 2.4 mm) (Fig. 2B).

As the calcium concentration of BHO was increased from 238 to 1,276 μM by the addition of calcium chloride, the colony diameter of P^+ cells was increased from 0.36 to 1.20 mm (as large as that on BHA at a 1,276 μM calcium concentration), abolishing gradually the unique colonial morphology of P^+ strains (Fig. 3). The addition of 10 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Sigma] to BHO prevented the P^+ strain from forming colonies, whereas the P^+ strain formed pinpoint colonies with the addition of 10 mM EGTA to BHA. A positive response in the CAD test with BHO was correlated with the presence of the plasmid and a number of virulence-associated properties, including crystal violet binding, autoagglutination, hydrophobicity, and mouse virulence (Table 1). Results similar to those obtained with BHO were obtained when agarose-based tryptic soy plating medium was used for the CAD test (data not shown).

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